

CHARACTERIZATION OF A NOVEL BACTERIAL DEHALOGENASE FROM COW DUNG

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CHARACTERIZATION OF A NOVEL BACTERIAL DEHALOGENASE
FROM COW DUNG

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Specially dedicated

To my lovely mother and my late father,

Siti Khadijah Draman & Ismail Jusoh

who gave me endless love, trust, constant encouragement and great source of
inspiration.

To my dear husband,

Dr. Arman Shah Abdullah

for being very understanding and supportive in keeping me going, enduring the
ups and downs during the completion of this thesis. I am truly thankful for having
you in my life.

To my adorable babies,

Ayra Shazia Putri Binti Arman Shah & Ayrin Shazwina Binti Arman Shah

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ABSTRACT

The large quantities of the halogenated compound for example 2,2-dichloropropionic acid (2,2DCP) in the environment may lead to health problems in humans and pollution due to their toxicity and recalcitrance, respectively. Interestingly, previous studies have indicated that cow dung was proven to degrade pollutants. Hence, such animals feeding on a daily diet of halogen contaminated forage may influence the microflora in their digestive tract. Bacterial species from cow dung able to utilize 2,2DCP is yet to be reported. Therefore, the purpose of this study was to isolate, identify and characterize dehalogenase bacteria from cow dung. Four bacteria were isolated which are SN1, SN2, SN3, and SN4. Strain SN1 was observed with rapid growth in 20 mM 2,2DCP liquid minimal media, and was used for further experiments such as growth in different concentration of 2,2DCP, High-performance liquid chromatography (HPLC), Biolog GENIII, 16S rRNA analysis, characterization of purified enzyme, kinetic analysis, Liquid Chromatography-Mass Spectrometry (LC-MS/MS) and amplification of dehalogenase gene. The growth of strain SN1 in various concentrations (10 mM, 20 mM, 30 mM and 40 mM) of the substance was evaluated. The study found the bacteria grew particularly well in 20 mM 2,2DCP with the highest chloride ion released ($39.5 \mu\text{molCl}^-/\text{mL}$) while exhibiting a remarkably short doubling time of 3.85 h. The utilization of 2,2DCP was also confirmed by detection of 20 mM 2,2DCP depletion in the growth medium containing strain SN1 measured using HPLC. The result showed 98.6 % utilization of 2,2DCP in the growth medium. Species identification via Biolog GENIII system and 16S rRNA analysis was performed and identified strain SN1 as *Bacillus cereus*. Further investigations on dehalogenase enzyme were done by using purified enzyme of *Bacillus cereus* SN1. The molecular weight of the purified enzyme was 25 kDa by SDS-PAGE. The enzyme characteristics revealed it was optimum at pH 6 and 30 °C. It also has low K_m value of 0.2 mM. The dehalogenase peptide was identified by LC-MS/MS with 18% sequence coverage to haloacid dehalogenase, *Bacillus cereus* (strain 03BB102). Moreover, Group I and Group II dehalogenase primers were used to amplify dehalogenase gene and the band only appeared for Group I. The dehalogenase gene fragment amplified was designated “DehSN1” and belongs to Group I dehalogenase since it has 75 % similarity with Group I dehalogenase (DehE). Five conserved residues were identified as Asn33, Tyr117, Cys42, Ala120 and Asp136. As a conclusion, this is the first reported case of a *Bacillus* sp. isolated from cow dung capable of utilizing 2,2DCP. Therefore, further assessment of its ability to degrade other types of haloalkanoic acids merits special consideration.

ABSTRAK

Kuantiti sebatian halogen yang besar seperti asid 2,2-dikloropropionik (2,2DCP) dalam persekitaran menyebabkan masalah kesihatan di kalangan manusia dan pencemaran disebabkan ianya toksik dan tegar. Menariknya, kajian lepas menyatakan bahawa najis lembu boleh menguraikan pencemaran. Oleh itu, haiwan yang memakan makanan hariannya yang tercemar dengan halogen, akan mempengaruhi mikroflora di dalam saluran penghadamannya. Spesis bakteria daripada najis lembu yang berupaya menggunakan 2,2DCP belum pernah di laporkan. Oleh itu, tujuan kajian ini adalah untuk mengasingkan, mengenalpasti dan mencirikan dehalogenase dari bakteria najis lembu. Empat jenis bakteria telah diasingkan seperti SN1, SN2, SN3 dan SN4. Strain SN1 mempamerkan pertumbuhan pantas dalam media minima cecair 20 mM 2,2DCP, dan ia digunakan untuk eksperimen seterusnya seperti pertumbuhan dalam kepekatan 2,2DCP yang berbeza, kromatografi cecair berprestasi tinggi (HPLC), Biolog GENIII, analisis 16S rRNA, pencirian enzim tulen, analisis kinetik, kromatografi cecair-spektrometri jisim (LC-MS/MS) dan amplifikasi gen dehalogenase. Pertumbuhan strain SN1 dalam variasi kepekatan bahan (10 mM, 20 mM, 30 mM and 40 mM) telah dikaji. Kajian ini mendapati bakteria tumbuh dengan baik dalam 20 mM 2,2DCP dengan perlepasan ion klorida yang tertinggi (39.5 $\mu\text{molCl}^-/\text{mL}$) disamping menunjukkan masa berganda yang singkat iaitu 3.85 jam. Penggunaan 2,2DCP juga dibuktikan dengan pengesanan pengurangan 20 mM 2,2DCP di dalam media pertumbuhan yang mengandungi strain SN1 yang diukur dengan HPLC. Hasil menunjukkan penggunaan 2,2DCP adalah sebanyak 98.6 % di dalam media pertumbuhan. Pengenalpastian spesis melalui sistem Biolog GENIII dan analisis 16S rRNA telah dijalankan dan strain SN1 dikenalpasti sebagai *Bacillus cereus*. Kajian selanjutnya terhadap enzim dehalogenase adalah dengan menggunakan enzim tulen dari *Bacillus cereus* SN1. Berat molekul enzim yang telah ditulenkan adalah 25 kDa melalui SDS-PAGE. Ciri-ciri enzim menunjukkan ia optima pada pH 6 dan 30 °C. Ia juga mempunyai nilai K_m yang rendah iaitu 0.2 mM. Peptida dehalogenase dikenalpasti melalui LC-MS/MS dengan liputan jujukan sebanyak 18 % dengan *Bacillus cereus* (strain 03BB102). Selain itu, primer dehalogenase Kumpulan I dan Kumpulan II digunakan untuk amplifikasi dehalogenase dan jalur hanya muncul pada Kumpulan I. Gen dehalogenase yang telah diamplifikasi dinamakan "DehSN1" dan ia tergolong dalam dehalogenase Kumpulan I memandangkan ia mempunyai persamaan 75 % dengan dehalogenase Kumpulan I (DehE). 5 residu telah dikenalpasti sebagai Asn33, Tyr117, Cys42, Ala120 dan Asp136. Sebagai kesimpulannya, ini adalah laporan yang pertama mengenai *Bacillus* sp. yang diasingkan daripada najis lembu yang mampu menggunakan 2,2DCP. Oleh itu, kajian selanjutnya perlu dipertimbangkan secara khusus tentang kebolehannya untuk menguraikan asid haloalkanoik berlainan jenis.

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LIST OF ABBREVIATIONS

2,2DCP	-	2,2-dichloropropionic acid
BLAST	-	Basic Local Alignment Search Tool
<i>et al.</i>	-	and friends
PCR	-	Polymerase Chain Reaction
<i>sp.</i>	-	Species
bp	-	Base pairs
rpm	-	Renovation per minute
nm	-	nanometre

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CHAPTER 1

INTRODUCTION

1.1 Background of the Study

The ever growing human population has resulted in the mass production of a myriad of halogenated compounds pertinently used in pharmaceutical and agricultural management (herbicides, fungicides, insecticides) as well as intermediates in organic solvents etc. However, the large presence of such substances in the environment may lead to undesirable consequences pertaining to health problems in humans and pollution due to their toxicity and recalcitrance, respectively (Pee and Unversucht, 2003). This problem has been further exacerbated by the fact that such halogenated chemicals i.e. 2,2-dichloropropionic acid (2,2DCP) has been extensively use as the prevailing active components in the production of herbicides (Dalapon) for weed management in agricultural practices. Pertinently, it has been indicated in literature that the heavy reliance on the chemical means to control weed in agricultural sectors may cause long term adverse effects to our existing fragile ecosystem (Reyes-Franco *et al.*, 2006; Srivinasan *et al.*, 2009). Therefore, alternative methods that would overcome such disadvantages need to be suggested. In this context, the enzymatic degradation of 2,2-dichloropropionic acid using dehalogenases may prove to be a promising alternative means to the prevailing chemical route to control weed in agriculture. The continuing search for novel biocatalysts is a matter of great interest as such only a fraction of the microbial species present in nature has been reported, encompassing only 0.2 to 0.6 % bacteria, 5 % fungi and a maximum of 24 % of known

algae (Wubbolts *et al.*, 2000). Therefore, such available wealth of undiscovered microorganisms out there has led in concerted efforts in search of novel microorganism with “unnatural” abilities to synthesize compounds that are not normally produced by “normal metabolic” routes (Adamczak and Krishna, 2004; Gomes and Steiner, 2004).

Hence, the hunt for such biocatalysts specific for a particular application remains a challenge. To date, two common approaches are used to select microorganisms suited for a particular task. The first approach involves screening for novel microorganisms by taking advantage of the largely unexplored biodiversity; and the second option is screening for new activities amongst the existing microorganisms. These approaches may also include works in the improvement of catalytic properties of known enzymes through protein engineering, either through molecular biology or direct protein modification (Hamid *et al.*, 2011). Moreover, chances of finding enzymes with extraordinary properties is fairly reasonable considering numerous reports pertaining to microbial isolation studies (Jing *et al.*, 2008a; Roslan *et al.*, 2011; Abel *et al.*, 2012b; Bagherbaigi *et al.*, 2013; Niknam *et al.*, 2014). Such findings have since open doors on explorations of different properties of dehalogenases from countless microorganisms capable of hydrolyzing 2,2-dichloropropionic acid (Jing *et al.*, 2008a; Roslan *et al.*, 2011; Abel *et al.*, 2012b; Bagherbaigi *et al.*, 2013; Niknam *et al.*, 2014).

Over the years, microorganisms displaying interesting properties have been isolated from extreme environments *viz.* high salt concentration or temperature as well as in polluted water bodies (Edbieb *et al.*, 2016; Hamid *et al.*, 2010a). Interestingly, several studies have indicated that the dung of a cow may prove to be a rich source of pollutant degrading microflora bacteria (Joshi and Pandey, 2011; Geetha and Fulekar, 2008; Randhawa and Kullar, 2011). In effect, previous studies have shown that the major portion in the daily diet of ruminant animals mostly consisted of forage (Hendriks, 2012; Camboim *et al.*, 2012a, 2012b) which may contain naturally occurring halogenated compounds (Siuda and De Bernardis, 1973; Fuge, 1988). Hence, it could not be ruled out that such animals feeding on a daily diet of halogen contaminated forage may inadvertently influence the microflora in their digestive tract.

Pertinently, recent studies have supported the hypothesis that the microflora in the digestive tract of cattle may develop special adaptations to counteract the effects of halogenated chemicals in their diet (Singh and Fulekar, 2007; Hendriks, 2012; Camboim *et al.*, 2012a, 2012b; Arunkumar and Chandrasekaran, 2013).

Reports by Hendriks (2012) and Camboim *et al.* (2012a) indicated that bacteria found in the digestive tract of herbivores which fed on naturally occurring halogenated compound monofluoroacetate in gifblaar (*Dichapetalum cymosum*) were effective in treating cows affected by the toxicity of monofluoroacetate (Hendriks, 2012; Camboim *et al.*, 2012a, 2012b). A previous work by Singh and Fulekar (2007) described several microorganisms namely *Pseudomonas* sp., *Streptococcus* sp., *Sarcina* sp., *Escherichia coli*, *Penicillium* sp., *Rhizopus* sp., *Mucor* sp. and *Nocardia* sp. isolated from the dung of cow were effective in degrading phenol. Correspondingly, endosulfan (Arunkumar and Chandrasekaran, 2013) and palm oil degrading microorganisms (Ojonoma and Udeme, 2014) isolated from similar sources have been reported. Therefore, this study will be focussing in the ability of microorganisms that will be isolated from cow dung to utilize 2,2-dichloropropionic acid as a carbon source and to characterise further the isolate.

1.2 Problem Statement

Halogenated compounds are commonly used as herbicides, pesticides, insecticides and antibiotics. The extensive use of halogenated compounds led to harmful effects on human and natural environment because of their toxicity and difficult to be degraded. Microorganisms cause natural degradation of the halogenated compounds to become less toxic compounds. Nevertheless, natural bioremediation is a slow process and needs to be enhanced by the action of the potential microorganisms. Example of naturally occurring halogenated compound, monofluoroacetate found in the plant, gifblaar (*Dichapetalum cymosum*) in the South African which resulted in acute death of ruminants, mostly cattle (Minnaar *et al.*, 2000). The young leaves of this plant

are most toxic during spring and autumn. The lethal oral dose for cattle is $0.15 \text{ mg (kg bodyweight)}^{-1}$. Therefore, the bacteria were used to reduce toxicity of monofluoroacetate (Hendriks, 2012). In addition, cow dung has potential bacteria that can degrade pollutants especially pesticides (Randhawa and Kullar, 2011). Current research reported that cow dung have the great influences to degrade crude oil and pesticides (Ikuesan *et al.*, 2015; Khan and Manchur, 2015).

Considering the abundance of forage fed cattle in the rural areas of Peninsular Malaysia, specific studies on isolating dehalogenase producing microbes from cow dung merits certain consideration. This present investigation attempted to isolate, characterize and identify such microorganism from the dung of free range cow capable of utilizing 2,2-dichloropropionic acid as sole source of carbon, consequently analyse and identify its dehalogenase. It is pertinent to highlight here that isolation studies on bacterial species from cow dung able to utilize 2,2-dichloropropionic acid is yet to be reported in Malaysia.

1.3 Objectives

- i. To isolate bacteria that capable to degrade 2,2-dichloropropionic acid from cow dung.
- ii. To identify and characterize the isolated bacteria based on growth experiment on different concentration of 2,2-dichloropropionic acid (2,2DCP), Biolog GEN III MicroPlate and molecular analysis.
- iii. To purify and characterize the dehalogenase by enzyme assay analysis, possibly to identify new kind dehalogenase.

1.4 Scope of Study

The work involves isolation of the bacteria from cow dung followed by bacterial identification and growth establishment on 2,2-dichloropropionic acid as a carbon source. The best colony grow on 2,2-dichloropropionic acid will be further analysed by the preparation of the crude extract from the cells grow on 2,2-dichloropropionic acid and assay the dehalogenase. It is hope that new kind of dehalogenases will be isolated from this study.

1.5 Significances and Original Contributions of This Study

A few studies of using cow dung to degrade some of pollutants such as benzene, phenol and pesticide were reported by Singh and Fulekar (2010), Singh and Fulekar (2009) and Geetha and Fulekar (2008) respectively. Yet, no research of using cow dung to degrade halogenated compound or 2,2-dichloropropionic acid was reported. Therefore, this research was focused on identifying the bacteria from cow dung that can degrade 2,2-dichloropropionic acid. This study also provides new knowledge on the degradation of α -haloalkanoic acids through the use of an exceptional dehalogenase producing bacteria. The properties of the α -haloalkanoic acid dehalogenase isolated in this study and 2,2-dichloropropionic acid product were evaluated which were important to recognize their catalytic properties. Amplification and analysis of α -haloalkanoic acid dehalogenase gene sequence was carried out from the isolated bacteria using Group I and Group II primers proposed by Hill *et al.* (1999). Thus, the group of dehalogenases was identified and the conserved gene sequence were known that was important for identifying key residues that responsible for dehalogenating capability. The information here, may be valuable in the future for precise prediction of the interaction between the active sites residues of the α -dehalogenase isolated from this novel bacteria with 2,2-dichloropropionic acid.

This research will contribute to save the cost of current bioremediation method by using the cow dung which is readily available. This research will reveal ability of halogenated compound to be degraded enzymatically, and to reduce its toxicity. The cow dung is highly potential to bio-remediate many pollutants because it contains various beneficial microorganisms to degrade pollutants. Therefore, the adverse effect of pollutants on environment can be reduced and save the future.

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